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Purification and Properties of *Penicillium* Glucose 6-Phosphate Dehydrogenase

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1. Glucose 6-phosphate dehydrogenase was isolated and partially purified from a thermophilic fungus, *Penicillium duponti*, and a mesophilic fungus, *Penicillium notatum*. 2. The molecular weight of the *P. duponti* enzyme was found to be 120000±10000 by gel-filtration and sucrose-density-gradient-centrifugation techniques. No $NADP^+$- or glucose 6-phosphate-induced change in molecular weight could be demonstrated. 3. Glucose 6-phosphate dehydrogenase from the thermophilic fungus was more heat-stable than that from the mesophile. Glucose 6-phosphate, but not $NADP^+$, protected the enzyme from both the thermophile and the mesophile from thermal inactivation. 4. The K_m values determined for glucose 6-phosphate dehydrogenase from the thermophile *P. duponti* were 4.3×10^{-5} M-$NADP^+$ and 1.6×10^{-4} M-glucose 6-phosphate; for the enzyme from the mesophile *P. notatum* the values were 6.2×10^{-5} M-$NADP^+$ and 2.5×10^{-4} M-glucose 6-phosphate. 5. Inhibition by NADPH was competitive with respect to both $NADP^+$ and glucose 6-phosphate for both the *P. duponti* and *P. notatum* enzymes. The inhibition pattern indicated a rapid-equilibrium random mechanism, which may or may not involve a dead-end enzyme–$NADP^+$–6-phosphogluconolactone complex; however, a compulsory-order mechanism that is consistent with all the results is proposed. 6. The activation energies for the *P. duponti* and *P. notatum* glucose 6-phosphate dehydrogenases were 40.2 and 41.4 kJ·mol^{-1} (9.6 and 9.9 kcal·mol^{-1}) respectively. 7. Palmitoyl-CoA inhibited *P. duponti* glucose 6-phosphate dehydrogenase and gave an inhibition constant of 5×10^{-6} M. 8. *Penicillium* glucose 6-phosphate dehydrogenase had a high degree of substrate and coenzyme specificity.

All the evidence available indicates that the metabolic processes in thermophilic organisms are no different from those found in mesophilic organisms (Farrell & Campbell, 1969). Consequently, there has been considerable speculation on the nature of the proteins present in organisms that grow and metabolize at high temperatures. Although the thermophilic bacteria and algae have been studied extensively (Farrell & Campbell, 1969), there has been very limited research into thermophilic fungi (Cooney & Emerson, 1964; Mumma *et al.*, 1970; Broad & Shepherd, 1970). Except for the alga *Cyanidium caldarium*, the thermophilic fungi are the only eucaryotic organisms that exhibit this adaptation to growth at elevated temperatures (Brock, 1967).

In studies of bacteria it has been found that the enzymes of thermophiles are more resistant to denaturation than enzymes from mesophiles (Amelunxen, 1966; Saunders & Campbell, 1966). It is possible that a certain amount of structural flexibility has been sacrificed to achieve this additional stability, and that, as a result, enzymes from thermophiles may be catalytically less efficient (Brock, 1967). Support for this suggestion came from Brock's (1967) summary of growth rates of various bacteria at their optimum temperatures when plotted according to the Arrhenius equation; his results showed that thermophiles do not grow as fast at their optimum temperatures as one would predict on theoretical grounds. It was therefore decided to determine whether the thermostability and kinetics of enzymes from thermophilic fungi are different from those of their mesophilic counterparts. The present paper describes physical and kinetic properties of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) isolated from the thermophilic and mesophilic fungi *Penicillium duponti* and *Penicillium notatum* respectively.

Materials and Methods

Materials

$NADP^+$, NAD^+, NADPH, NADH, glucose 6-phosphate, D-glucosamine 6-phosphate, D-galactosamine 6-phosphate, D-mannose 6-phosphate, 6-phospho-D-gluconate, palmitoyl-CoA, *p*-chloromercuribenzoate, fumarase (pig heart), catalase (bovine liver), peroxidase (horse-radish, type II), cytochrome *c* (horse heart), triose phosphate isomerase, α-glycerophosphate dehydrogenase, phosphoglycerate kinase and glucose oxidase were

obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Hexokinase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase (muscle) and glucose 6-phosphate dehydrogenase (yeast) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Bovine serum albumin was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Protamine sulphate was a product of British Drug Houses, Poole, Dorset, U.K. Tween 80 was obtained from Atlas Powder Co., Wilmington, Del., U.S.A. Chloramphenicol was obtained from Parke, Davis and Co., Sydney, Australia. DEAE- and CM-cellulose were Whatman DE11 and CM22, from W. and R. Balston Ltd., Maidstone, Kent, U.K. Blue Dextran 2000 and Sephadex G-100 and G-200 were supplied by Pharmacia, Uppsala, Sweden. Other chemicals were of analytical-reagent grade. Alcohol dehydrogenase was a crude supernatant prepared from autolysed yeast cells by centrifuging the lysate at 12000*g* for 15min; it also contained aldolase and glucose 6-phosphate dehydrogenase activity. Crystalline horse oxyhaemoglobin was prepared by the method of Heidelberger (1922) as described by Lemburg & Legge (1949).

Cells and culture conditions

P. duponti, *Humicola lanuginosa* and *Chaetomium thermophile* var. *coprophile* were derived from cultures obtained from Dr. R. Emerson, Botany Department, University of California, Berkeley, San Francisco, Calif., U.S.A. *P. notatum*, *Mucor miehei*, *Penicillium chrysogenum* and *Penicillium urticae* were obtained from Northern Research Regional Laboratories, Peoria, Ill., U.S.A. All the fungi were propagated on yeast–glucose agar of the following composition: powdered yeast extract, 5g; glucose, 10g; agar, 20g; tap water, 1 litre; 1M-HCl to give pH5.0. The methods for transferring spores and inoculating media were as described previously (Gaucher & Shepherd, 1968). Cultures of thermophiles and mesophiles were maintained at 45° and 28°C respectively. It was found that for continuous growth on solid media the relative humidity must be above 30%.

Submerged cultures were prepared in the modified Czapek–Dox medium, as described by Gaucher & Shepherd (1968), except that the concentration of glucose was 1% (w/v). For *P. duponti* cells, spores from a slant culture were added to 500ml of autoclaved medium in a 1-litre Erlenmeyer flask; this flask was modified with three vertical flutes to improve aeration. After shaking for 24h at 45°C on a New Brunswick Gyrotory Shaker (250rev./min), this 'starter culture' was transferred to 3.5 litres of medium in a 5-litre fluted Erlenmeyer flask; this medium had been preheated to 45°C, and contained 50mg of chloramphenicol to prevent bacterial contamination. The cells were harvested after shaking for 48h at 45°C. For *P. notatum* cells, 1 litre of autoclaved medium in a 2-litre Erlenmeyer flask was inoculated with a spore suspension from a slant culture and was shaken at 28°C for 36h before harvesting.

Buffers

Constant-ionic-strength buffers were prepared as described by Long (1961). The buffer used for assay of glucose 6-phosphate dehydrogenase was tris–HCl, pH8.0 (25°C), $I = 0.1$, containing 10mM-$MgCl_2$. The phosphate buffer ($KH_2PO_4 - Na_2HPO_4$) used in the purification of glucose 6-phosphate dehydrogenase, up to and including the Sephadex column step, was pH6.8, $I = 0.05$, containing 1mM-EDTA; the sodium citrate buffer was pH5.4, 0.01M, containing 0.1mM-EDTA. The Sephadex G-200 column was equilibrated with phosphate buffer ($KH_2PO_4 - Na_2HPO_4$), pH7.0, $I = 0.01$, containing 0.2M-ammonium sulphate.

Methods

Assay of glucose 6-phosphate dehydrogenase. Glucose 6-phosphate dehydrogenase activity was measured as described previously (Broad & Shepherd, 1970) in tris–HCl buffer, pH8.0, $I = 0.09$, containing 9mM-$MgCl_2$ (final concentration) in a total volume of 1.12ml. A unit of enzyme is defined as the amount of enzyme that will reduce 1μmol of $NADP^+$/min at 25°C.

Determination of protein. Protein concentration was measured by a modification (Eggstein & Kreutz, 1967) of the method of Lowry *et al.* (1951), with sodium citrate being used instead of sodium tartrate. When there was interference with this method, protein was estimated by its E_{260} and E_{280} (Warburg & Christian, 1931).

Preparation of cell-free extracts. Cell-free extracts were prepared from the fungal cells by sonication and centrifugation as described by Gaucher & Shepherd (1968).

Preparation of glucose 6-phosphate dehydrogenase from P. duponti. Glucose 6-phosphate dehydrogenase was purified by a modification of the procedure developed by Broad & Shepherd (1970). All operations were carried out at 0–4°C.

Protamine sulphate (0.1g/50g wet wt. of cells) was dissolved in 5–10ml of the phosphate buffer and was added to the cell-free extract; after stirring for 10min, the suspension was centrifuged for 10min at 20000*g*. The supernatant was adjusted to 45% saturation with solid $(NH_4)_2SO_4$ (277g/l of supernatant) and centrifuged (10min at 20000*g*); the pellet was discarded. The $(NH_4)_2SO_4$ concentration of the supernatant was

increased to 60% saturation [by adding 99g of solid $(NH_4)_2SO_4$/l to the 45%-saturated-$(NH_4)_2SO_4$ supernatant], and the pellet formed after centrifuging (10min at 20000*g*) was dissolved in 3–5ml of phosphate buffer. This solution was layered on to a Sephadex G-100 column (2.5cm×40cm), which had been equilibrated with phosphate buffer, and the column was eluted at a rate of 30ml/h. Fractions (5ml) were collected with an LKB fraction collector and those fractions with more than 50% of the activity of the peak fraction were pooled (total volume 40 or 45ml) and concentrated to about 5ml in an Amicon Ultrafiltration Cell with a PM-10 membrane. The concentrated solution was diluted to 50ml with citrate buffer and re-concentrated to 5ml; this dilution and concentration procedure was repeated and the final volume of concentrate after rinsing the apparatus was about 10ml. The enzyme solution was then applied to a CM-cellulose column (1.6cm× 44cm), which had been equilibrated with citrate buffer, and the column was eluted at a rate of 25ml/h with a 500ml linear gradient of 0–0.08M-$(NH_4)_2SO_4$ in citrate buffer. Two peaks of glucose 6-phosphate dehydrogenase activity were obtained (Fig. 2). The active fractions from both peaks were pooled together (120ml) and concentrated by ultrafiltration to 10ml, diluted to 50ml with citrate buffer and re-concentrated to 30ml, then re-chromatographed on CM-cellulose under the conditions described above. From this second column only one region of activity was recovered, and the fractions containing more than 50% of the activity of the peak fraction were pooled (30ml), concentrated by ultrafiltration to 5–10ml and stored at 4°C.

Preparation of glucose 6-phosphate dehydrogenase from P. notatum. The above procedure was also used for the preparation of *P. notatum* glucose 6-phosphate dehydrogenase, but it was followed only as far as the gel-filtration step. This yielded a preparation that was 40-fold purified and had a specific activity of 5.1 units/mg of protein.

Polyacrylamide-gel electrophoresis. Electrophoresis was carried out by a modification of the method of Ornstein & Davis (1964); 7.5% gels were run in tris–glycine buffer at pH8.4. Protein was stained with Amido Schwarz, and glucose 6-phosphate dehydrogenase activity was detected by the method of Brewer & Ashworth (1969).

Sephadex G-200 gel filtration. A column (2.4cm× 48cm) of Sephadex G-200 was calibrated for molecular-weight determination by the technique described by Andrews (1964). Samples were applied in a volume of 1ml, and a constant flow rate of 8ml/h was used for elution. Fractions containing 32 drops were collected for analysis. When the column was not in use, 0.03% NaN_3 in the phosphate buffer protected it from bacterial contamination.

The elution volume of *P. duponti* glucose 6-phosphate dehydrogenase was determined at four different stages of purification, and as a check on the calibration, marker proteins were chromatographed in various combinations several times during the course of the study. Blue Dextran (2mg) was included in all samples except for those containing *P. duponti* glucose 6-phosphate dehydrogenase (the purified enzyme bound strongly to the dextran).

Distribution coefficients (K_d) were calculated from the basic equation given by Gelotte (1960): $K_d = (V_e - V_0)/V_i$, where V_e is the peak effluent volume, V_0 is the void volume (elution volume of Blue Dextran), and V_i is the volume of solvent within the gel phase (elution volume of ferricyanide minus V_0).

K_d was used in two ways; first by the method of Andrews (1964) to obtain molecular weight, and secondly by the method of Ackers (1964) to derive the Stokes radius.

Sucrose-density-gradient centrifugation. The sedimentation coefficient of *P. duponti* glucose 6-phosphate dehydrogenase was determined by the method of Martin & Ames (1961). Centrifugation was carried out in the SW 50.1 rotor in a Beckman model L2-65B ultracentrifuge at 2°C.

Assay of marker proteins. Cytochrome *c* and haemoglobin were measured by their absorption at 410nm, and bovine serum albumin and Blue Dextran were measured at 280nm. Conditions for the assay of marker enzymes were those given by Bergmeyer (1963).

Kinetic analyses. The *P. duponti* and *P. notatum* glucose 6-phosphate dehydrogenase preparations used for the kinetic analysis had specific activities of 66 units/mg and 5.1 units/mg of protein respectively. All the lines obtained in these studies were derived by the method of least squares with an Olivetti Programma 101 desk-top computer. Initial velocities are expressed as μmol of NADPH produced/min per ml of enzyme solution at 25°C.

Results

Growth characteristics of P. duponti

The growth characteristics of *P. duponti* grown in submerged culture at 45°C in a 5-litre Erlenmeyer flask are shown in Fig. 1. A yield of 5g dry wt./litre corresponded to about 25g wet wt. of cells/litre. Since the specific activity of glucose 6-phosphate dehydrogenase remained constant throughout 48h of growth, the cells were harvested early in the stationary phase.

If the medium contained more or less than 1% (w/v) of glucose, the cell yield was decreased. Neither the concentration of glucose nor the addition of chloramphenicol to the medium affected the specific activity of the glucose 6-phosphate dehydrogenase.

Purification of glucose 6-phosphate dehydrogenase from P. duponti

Typical results of the purification scheme are given in Table 1. The specific activity of the purified enzyme was 66 units/mg; it should be noted that Broad & Shepherd (1970) reported specific activities that were 10 times too high for the same degree of purification. The enzyme was always eluted from the first CM-cellulose column as two activity peaks; most of the protein was excluded from this column and did not contain any glucose 6-phosphate dehydrogenase activity. A typical elution profile is given in Fig. 2. When the active fractions of both peaks were pooled then re-chromatographed in the same manner, the one activity peak obtained appeared in the region of peak II of the first column. The two activity peaks from the first CM-cellulose column were each concentrated by ultrafiltration and then examined by gel-filtration and heat-inactivation studies in an attempt to distinguish them by some physical property. Their elution volumes from gel filtration on a Sephadex G-200 column were identical, but the recovery of peak I enzyme from the gel column was much lower than that of peak II enzyme. In a preliminary investigation, peak I enzyme was inactivated more quickly at 53°C than was peak II enzyme; both were protected from inactivation by glucose 6-phosphate. However, the specific activities of the two peaks differed: after concentration, peak I was 16.9 units/mg and peak II was 11.8 units/mg (see section on heat inactivation). The final enzyme solution (66 units/mg) was examined for purity by polyacrylamide-gel electrophoresis, and glucose 6-phosphate dehydrogenase activity was located in a band representing about 50% of the total protein.

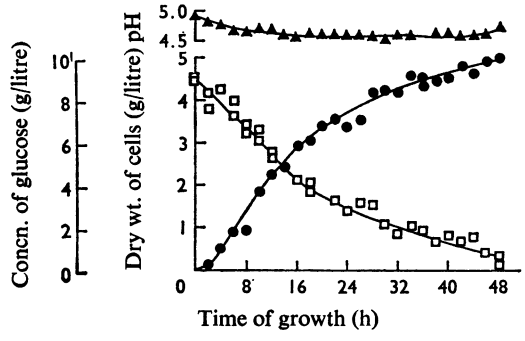

Fig. 1. *Growth characteristics of P. duponti in submerged culture at 45°C*

P. duponti was grown in a 5-litre fluted Erlenmeyer flask (as described in the Materials and Methods section). The experiment was carried out with four separate cultures for the 0–12h, 12–24h, 24–36h and 36–48h time-periods; 50ml samples were removed at 2h intervals and analysed for: ●, dry weight of cells (Gaucher & Shepherd, 1968); □, glucose concentration in the medium (Huggett & Nixon, 1965); ▲, pH and the specific activity of glucose 6-phosphate dehydrogenase in the cells (not shown).

Properties of the glucose 6-phosphate dehydrogenases

Stability. The purified enzyme from *P. duponti* was very unstable (half-life = 24h at 4°C in citrate buffer) except in the presence of glucose 6-phosphate.

The enzyme was more stable in a relatively impure state. The 60%-satd.-$(NH_4)_2SO_4$ pellet fraction could be stored for over a week in citrate buffer at −20° or −40°C without loss of activity; at 4°C and room temperature for 24h, this fraction lost 30 and 50% of its activity respectively. $(NH_4)_2SO_4$ (10%) gave

Table 1. *Purification of P. duponti glucose 6-phosphate dehydrogenase*

For details see the text.

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Cell-free extract	600	348	2820	0.12	—
Protamine sulphate supernatant	600	354	1680	0.21	100
45%-satd.-$(NH_4)_2SO_4$ supernatant	635	375	1460	0.26	100
60%-satd.-$(NH_4)_2SO_4$ supernatant	20	406	390	1.04	100
Sephadex G-100 eluate	40	404	158	2.56	100
First CM-cellulose eluate	120	260	51	5.1	79
Concentrated eluate	30	261	21	12.5	79
Second CM-cellulose eluate (concentrated)	7	111	1.68	66	34

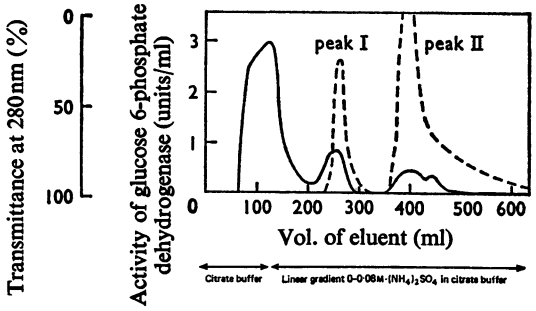

Fig. 2. *Elution profile for protein and glucose 6-phosphate dehydrogenase when first chromatographed on a CM-cellulose column during purification of the enzyme from P. duponti*

For details see the text. ——, Transmission at 280nm (%) (LKB Uvicord II); ----, glucose 6-phosphate dehydrogenase activity in units/ml of eluent.

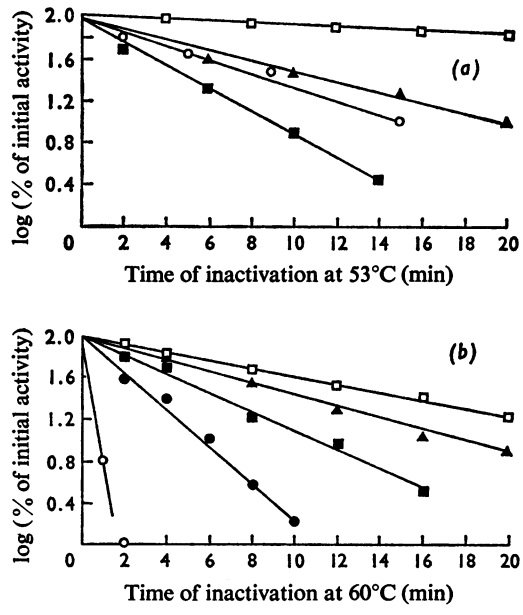

Fig. 3. *Heat inactivation of P. duponti and P. notatum glucose 6-phosphate dehydrogenases at 53°C and 60°C*

For the inactivation, the samples were diluted to an activity of 0.24 unit/ml in the assay buffer (pH8.0). For other details see the text. (*a*) At 53°C, *P. duponti* glucose 6-phosphate dehydrogenase: □, specific activity, 0.11 (cell-free extract), 0.35 (105000*g*, 90min supernatant from cell-free extract), 1.12 (dialysed 60% pellet fraction), or 66 units/mg (purified enzyme). *P. notatum* glucose 6-phosphate dehydrogenase: ○, 0.09 unit/mg (cell-free extract); ▲, 0.28 unit/mg (105000*g*, 90min supernatant from cell-free extract); ■, 0.98 unit/mg (dialysed 60% pellet fraction). (*b*) At 60°C, *P. duponti* glucose 6-phosphate: □, specific activity 0.11 unit/mg; ▲, 0.35 unit/mg; ■, 1.12 units/mg; ●, 66 units/mg. *P. notatum* glucose 6-phosphate dehydrogenase: ○, 0.09 unit/mg.

almost complete stability for 1–2 days at 4°C when the specific activity of the fraction was below about 10 units/mg of protein.

When the stability and activity of the 60%-satd.-$(NH_4)_2SO_4$ pellet fraction was studied as a function of pH in buffers of constant ionic strength, stability was found to be maximal between pH values of 5.4 and 7.0 (citrate and phosphate buffers), in contrast to a pH–activity optimum in the range pH8–9.

Heat inactivation. Purified glucose 6-phosphate dehydrogenase from *P. duponti* had a half-life in the assay buffer of 4.8h at 45°C, its temperature of synthesis. Fig. 3 shows that (1) as the specific activity of the enzyme from both *P. duponti* and *P. notatum* increases, the half-life decreases, (2) that thermal inactivation of glucose 6-phosphate dehydrogenase appears to follow first-order kinetics, and (3) that the enzyme from *P. duponti* is more heat stable than that from *P. notatum*; for example, at 53°C the half-life of *P. duponti* glucose 6-phosphate dehydrogenase (specific activity = 1.12 units/mg) is 36min, but for the *P. notatum* enzyme (specific activity = 0.98 unit/mg) the half-life is 2.5min.

Glucose 6-phosphate protects the enzyme from thermal inactivation (Fig. 4*a*); for example, the half-life of *P. duponti* glucose 6-phosphate dehydrogenase (specific activity = 1.12 units/mg) at 60°C is increased from 3min to 100min by the addition of glucose 6-phosphate. The inactivation of *P. duponti* glucose 6-phosphate dehydrogenase (specific activity = 66 units/mg) was examined at various concentrations of glucose 6-phosphate by the procedure of Burton (1951). The rate constant of inactivation, obtained from the equation $k = 1/t \cdot \ln(x_0/x)$, was plotted against the concentration of glucose 6-phosphate (Fig. 4*b*). x is the enzyme activity remaining after incubation at 60°C with the appropriate concentration of glucose 6-phosphate, x_0 is the corresponding activity remaining in a control kept at 0°C, and t is the time of incubation (10min) at 60°C. All inactivations were carried out in the standard assay buffer. If k_0 is the rate constant of inactivation in the absence of glucose 6-phosphate and k_x is the minimum rate constant of inactivation, then by analogy with the Michaelis constant, the protection constant (π_g) is the concentration of

glucose 6-phosphate at which $k = \frac{1}{2}(k_0 + k_x)$. From Fig. 4(*b*), $\pi_g = 2.25 \times 10^{-3}$ M.

The heat stability of the enzyme was found to be a function of pH. At 53°C, the half-life of *P. duponti* glucose 6-phosphate dehydrogenase (specific activity = 66 units/mg) was 4min in tris–HCl buffer, pH8.0, $I = 0.1$, 8min in tris–HCl buffer, pH7.4, $I = 0.1$, and 50min in phosphate buffer, pH6.0, $I = 0.1$.

Reactivation could not be achieved by incubation of the inactivated enzyme with or without glucose 6-phosphate at 0° or 53°C, and neither the *P. duponti* nor the *P. notatum* enzyme was protected from heat inactivation by $NADP^+$. The rate of heat inactivation of the enzyme was not dependent on the concentration of enzyme inactivated. This difference in the thermostability of glucose 6-phosphate dehydrogenase from the thermophilic and mesophilic fungi is not confined to the two fungi selected for detailed study, as shown in Table 2; these other fungal glucose 6-phosphate dehydrogenases were also protected from heat inactivation by glucose 6-phosphate.

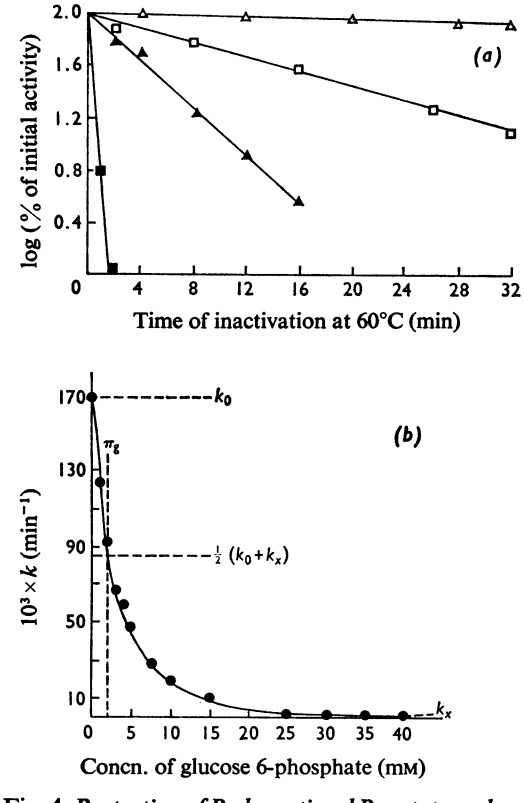

Fig. 4. *Protection of P. duponti and P. notatum glucose 6-phosphate dehydrogenases from heat inactivation at 60°C by glucose 6-phosphate*

For details see the text. (*a*) *P. duponti* glucose 6-phosphate dehydrogenase, 1.12 units/mg: △, +50mM-glucose 6-phosphate; ▲, no glucose 6-phosphate. *P. notatum* glucose 6-phosphate dehydrogenase, 0.98 unit/mg: □, +50mM-glucose 6-phosphate; ■, no glucose 6-phosphate. (*b*) Variation of the rate constant of inactivation, k (min^{-1}), with the concentration of glucose 6-phosphate for *P. duponti* glucose 6-phosphate dehydrogenase, 66 units/mg.

Inactivation by urea and p-chloromercuribenzoate. *P. duponti* glucose 6-phosphate dehydrogenase was inactivated by both urea and *p*-chloromercuribenzoate as shown in Table 3. Glucose 6-phosphate or $NADP^+$ gave partial protection from these inactivating agents.

Determination of molecular weight. The molecular weight of *P. duponti* glucose 6-phosphate dehydrogenase was estimated by gel-filtration and sucrose-density-gradient centrifugation by using the well-characterized proteins shown in Fig. 5 and Table 4. The gel filtration on Sephadex G-200 yielded a molecular weight of 126000 (Fig. 5*a*) and a Stokes radius of 4.4×10^{-7}cm (Fig. 5*b*) for the enzyme. An average sedimentation coefficient of 6.2S was obtained from the sucrose-density-gradient-centrifugation technique (Table 4). This value was used together with the Stokes radius to calculate the molecular weight (Siegel & Monty, 1966) by the formula $M = 6\pi N\eta as/(1-\bar{v}\rho)$, where M = molecular weight, N = Avogadro's number, η = viscosity of the medium, a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume and ρ = density of the medium. When the partial specific volume was assumed to be 0.725ml/g, a value selected by Martin & Ames (1961) as being representative of most proteins, the calculation yielded an average molecular weight of 113000. A molecular weight of 121000 was obtained if the apparent partial specific volume of 0.744ml/g derived by Yue *et al.* (1969) for yeast glucose 6-phosphate dehydrogenase was used.

Identical elution volumes were obtained with the *P. duponti* enzyme at all stages of purification, including each activity peak from the first CM-cellulose column, and the enzyme was eluted from the Sephadex column as a symmetrical activity peak. Yeast glucose 6-phosphate dehydrogenase was eluted from the Sephadex G-200 column slightly after the *P. duponti* enzyme, but sedimented in a sucrose density gradient at the same rate as the fungal enzyme.

The addition of either glucose 6-phosphate or $NADP^+$ to the gradients (0.1–1mM final concentration) did not alter the sedimentation rate of *P. duponti* glucose 6-phosphate dehydrogenase.

Initial-velocity results. Saturation curves for $NADP^+$ and glucose 6-phosphate were obtained for both *P. duponti* and *P. notatum* glucose 6-phosphate

Table 2. *Thermostability of glucose 6-phosphate dehydrogenases in extracts of thermophilic and mesophilic fungi*

The half-life of glucose 6-phosphate dehydrogenase in cell-free extracts (see the Materials and Methods section) of each fungus was determined at 53° and 60°C, in the presence and the absence of glucose 6-phosphate at a concentration of 50mM.

Organism	Specific activity (unit/mg)	Half-life at 53°C (min)		Half-life at 60°C (min)	
		−Glucose 6-phosphate	+Glucose 6-phosphate	−Glucose 6-phosphate	+Glucose 6-phosphate
Thermophiles					
P. duponti	0.11	40	—	8	100
M. miehei	0.10	20	120	2	15
H. lanuginosa	0.09	28	180	6	60
C. thermophile var. *coprophile*	0.07	32	200	5	62
Mesophiles					
P. notatum	0.09	4	60	<1	10
P. chrysogenum	0.10	3	55	<1	6
P. urticae	0.09	4	70	<1	15

Table 3. *Inactivation of P. duponti glucose 6-phosphate dehydrogenase by urea and p-chloromercuribenzoate*

The enzyme was incubated in a spectrophotometer cuvette at 20°C in the assay buffer containing 2.6M-urea or 0.1mM-*p*-chloromercuribenzoate and the substrate concentration indicated below in a total volume of 1ml. After inactivation for 1min in urea or 5min in *p*-chloromercuribenzoate, the residual activity was determined by adding glucose 6-phosphate and $NADP^+$ (in a volume of 70μl) to the cuvette. The results are expressed as a percentage of the activity in the absence of an inactivator. Enzyme A had a specific activity of 1 unit/mg (dialysed 60% pellet) and enzyme B had a specific activity of 66 units/mg.

Substrate	Activity remaining (%)			
	Urea		*p*-Chloromercuribenzoate	
	Enzyme A	Enzyme B	Enzyme A	Enzyme B
No substrate	53	70	18	50
Glucose 6-phosphate (0.93mM)	56	74	—	53
Glucose 6-phosphate (9.3mM)	80	92	93	92
$NADP^+$ (0.46mM)	49	71	—	69
$NADP^+$ (2.3mM)	63	81	65	82

Table 4. *Sucrose-density-gradient centrifugation of P. duponti glucose 6-phosphate dehydrogenase*

The $s_{20,w}$ of *P. duponti* glucose 6-phosphate dehydrogenase was obtained by multiplying R (the distance moved by the glucose 6-phosphate dehydrogenase divided by the distance moved by the standard protein) by the $s_{20,w}$ value of the standard. The molecular weights were calculated as described in the text. The numbers in parentheses refer to the number of determinations of R, and the variations in the value of R were less than 2%.

Standard protein	$s_{20,w}$ (S)	Reference	$R = \frac{\text{unknown}}{\text{standard}}$	Values calculated for glucose 6-phosphate dehydrogenase	
				$s_{20,w}$ (S)	Molecular weight
Catalase	11.3	Sumner & Gralen (1938)	0.60 (6)	6.81	124000
Alcohol dehydrogenase	7.61	Theorell & Bonnichsen (1951)	0.82 (4)	6.24	113600
Lactate dehydrogenase	7.35	Pesce *et al.* (1964)	0.86 (2)	6.29	114400
Haemoglobin	4.41	Svedberg & Pedersen (1940)	1.31 (4)	5.79	105400
Cytochrome *c*	2.1	Atlas & Farber (1956)	2.91 (2)	6.00	109100

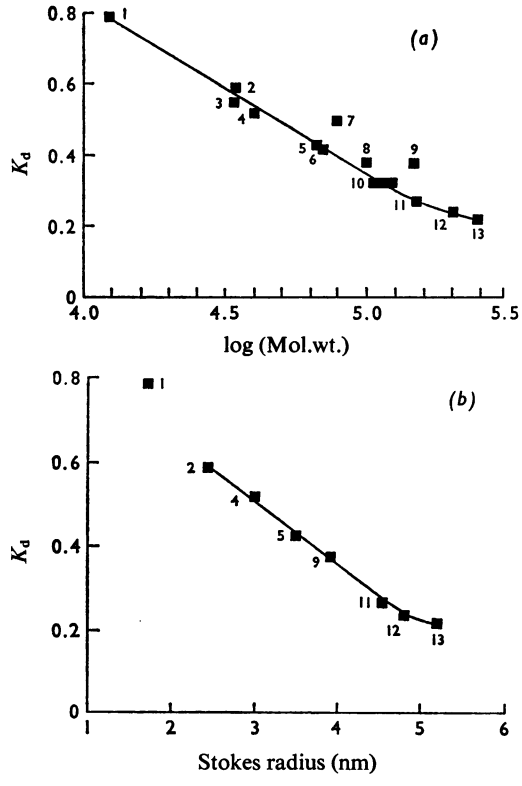

Fig. 5. *Molecular-weight determination by Sephadex G-200 gel filtration*

(*a*) Correlation of K_d with log (molecular weight). (*b*) Correlation of K_d with Stokes radius. Standard proteins were: 1, cytochrome *c* (Ackers, 1964; Margoliash & Lustgarten, 1962); 2, horse haemoglobin dimer (Ackers, 1964; Svedberg & Pedersen, 1940); 3, phosphoglycerate kinase (Bergmeyer, 1963); 4, peroxidase (Cecil & Ogston, 1951); 5, bovine serum albumin (Siegel & Monty, 1966; Florkin & Stotz, 1963); 6, yeast aldolase (Richards & Rutter, 1961); 7, α-glycerophosphate dehydrogenase (Bergmeyer, 1963); 8, hexokinase (Schulze *et al.*, 1966); 9, glyceraldehyde 3-phosphate dehydrogenase* (Taylor & Lowry, 1956; Jaenicke *et al.*, 1968); 10, yeast glucose 6-phosphate dehydrogenase* (Andrews, 1965; Yue *et al.*, 1969); 11, yeast alcohol dehydrogenase (Ackers, 1964; Dixon & Webb, 1964); 12, fumarase* (Massey, 1952); 13 catalase (Ackers, 1964; Dixon & Webb, 1964). The references given were the sources of molecular weight, Stokes radius and diffusion coefficient values. * denotes a Stokes radius (*a*) calculated from the diffusion coefficient by means of the equation, $a = kT/6\pi\eta D$, where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium and D is the diffusion coefficient. The K_d values have an error of ±0.01.

dehydrogenases as described in Figs. 6 and 8; these curves were hyperbolic and the same results gave linear double-reciprocal plots (Figs. 6 and 8*a*). Broad & Shepherd (1970) reported sigmoidal saturation curves and values of the Hill coefficient (*n*) greater than unity. These sigmoidal curves were, however, artificial because a logarithmic scale was used for the substrate concentration. The *n* values then reported were wrongly calculated.

The double-reciprocal plots in Figs. 6 and 8(*a*) show lines converging at a point slightly above the *x* axis. Secondary plots of these results (Figs. 7, 8*b* and 8*c*) were linear and yielded the following Michaelis constants: *P. duponti* enzyme, $K_m(NADP^+) = 4.3\times10^{-5}$M, K_m(glucose 6-phosphate) $= 1.6\times10^{-4}$M; *P. notatum* enzyme, $K_m(NADP^+) = 6.2\times10^{-5}$M, K_m(glucose 6-phosphate) $= 2.5\times10^{-4}$M.

Product inhibition. The inhibition of *P. duponti* and *P. notatum* glucose 6-phosphate dehydrogenases by NADPH was examined by measuring initial velocities at different concentrations of one substrate in the presence of NADPH and a constant non-saturating concentration of the other substrate (Figs. 9 and 10). The inhibition patterns were competitive with respect to both glucose 6-phosphate and $NADP^+$.

Dixon (1953) plots of the results in Figs. 9 and 10 gave the following inhibition constants for NADPH: *P. duponti* enzyme, 2.2×10^{-5}M when $NADP^+$ was varied, and 3.0×10^{-5}M when glucose 6-phosphate was varied; *P. notatum* enzyme, 2.6×10^{-5}M when $NADP^+$ was varied, and 3.1×10^{-5}M when glucose 6-phosphate was varied.

Inhibition by palmitoyl-CoA. The inhibition of *P. duponti* glucose 6-phosphate dehydrogenase by palmitoyl-CoA was found to be non-competitive with respect to both glucose 6-phosphate and $NADP^+$. Dixon (1953) plots of the results gave an inhibition constant of about 5×10^{-6}M-palmitoyl-CoA.

Specificity. NAD^+ was not reduced by *P. duponti* glucose 6-phosphate dehydrogenase, and neither NAD^+ nor NADH inhibited its activity. Glucose, D-glucosamine 6-phosphate, D-mannose 6-phosphate and D-galactose 6-phosphate were each tested as possible substrates and inhibitors of the enzyme, but none of them caused reduction of $NADP^+$ or inhibited glucose 6-phosphate dehydrogenase activity.

Activation energy. The activation energies (E_a) of the glucose 6-phosphate dehydrogenases from *P. duponti* and *P. notatum* were determined. The temperature of the cuvette chamber in the spectrophotometer was adjustable and the actual temperature was recorded by a sensor with an accuracy of ±1°C; this was checked with a thermometer in a cuvette kept in the cuvette chamber. The buffer was pre-heated to a temperature above that required so that when ice-cold enzyme and substrate were added, the final tempera-

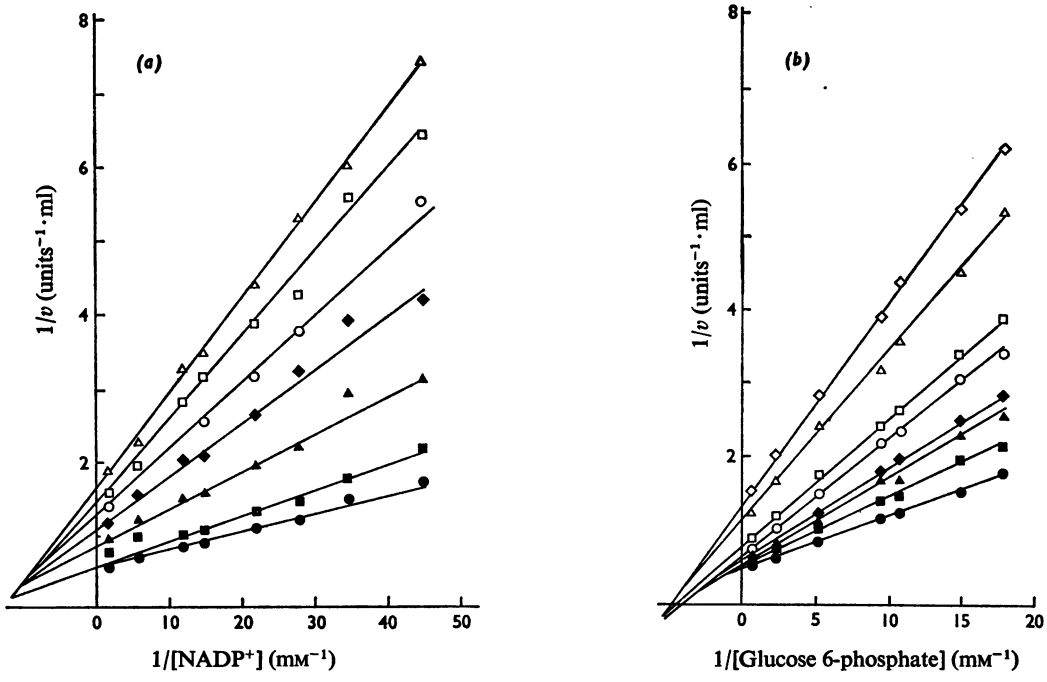

Fig. 6. *Double-reciprocal plots for P. duponti glucose 6-phosphate dehydrogenase*

(*a*) Variation of the initial rate (*v*) of NADPH production with $NADP^+$ concentration at different fixed concentrations of glucose 6-phosphate: ●, 0.89mM; ■, 0.45mM; ▲, 0.18mM; ◆, 0.11mM; ○, 89μM; □, 67μM; △, 56μM. (*b*) Variation of the initial rate (*v*) of NADPH production with glucose 6-phosphate concentration at different fixed concentrations of $NADP^+$: ●, 0.45mM; ■, 0.18mM; ▲, 89μM; ◆, 67μM; ○, 45μM; □, 36μM; △, 29μM; ◇, 22μM.

ture was close to that required. At the higher temperatures, saturation of the enzyme was checked by performing an assay at twice the normal substrate concentrations. The Arrhenius plots are shown in Fig. 11 and the E_a values are as follows: *P. duponti* enzyme, $E_a = 40.2 kJ \cdot mol^{-1}$ ($9.6 kcal \cdot mol^{-1}$); *P. notatum* enzyme, $E_a = 41.4 kJ \cdot mol^{-1}$ ($9.9 kcal \cdot mol^{-1}$).

Discussion

The obligate thermophilic and mesophilic organisms used for this study had no common growth temperature in the medium described; *P. notatum* grows in the temperature range 20°–35°C, whereas *P. duponti* grows between 37° and 58°C (Miller & Shepherd, 1972). This situation should be contrasted with that of thermophilic and mesophilic bacteria, where common temperatures of growth are generally found. It might be expected, therefore, that differences in the macromolecules from thermophiles and mesophiles may be greater in fungi than in bacteria.

A concentration of glucose greater or less than 1% resulted in a lower yield of cells; perhaps this is a result of catabolite repression of tricarboxylic acid-cycle enzymes (Flavell & Woodward, 1970). Bacterial contamination of the cultures could be prevented by the addition of chloramphenicol, since this antibiotic affected neither the growth of the cells nor the activity of glucose 6-phosphate dehydrogenase. The pH of the culture medium remained between 4.5 and 5.0 throughout growth (Fig. 1), and this would also minimize bacterial contamination.

The purified preparation of *P. duponti* glucose 6-phosphate dehydrogenase was not homogeneous. The instability of the purified enzyme presented the major difficulty in obtaining a better purification; however, the use of glucose 6-phosphate as a stabilizing agent during purification should overcome this problem.

Two regions of activity were obtained from the first CM-cellulose column (Fig. 2), but no significant differences in the physical properties of the enzyme from each peak could be detected. The differences observed could probably be accounted for by their

different specific activities. The purified enzyme was obtained from the second CM-cellulose column as one activity peak, and this preparation yielded only one activity band on electrophoresis in polyacrylamide gels. Although we have no convincing evidence for two different forms of *P. duponti* glucose 6-phosphate dehydrogenase, Domagk *et al.* (1969) have reported evidence for two different glucose 6-phosphate dehydrogenases in *Candida utilis*.

P. duponti glucose 6-phosphate dehydrogenase was found to have a molecular weight of about 120000, which is similar to that of the glucose 6-phosphate dehydrogenase apoenzymes from other sources. The sedimentation coefficient of 6.2S obtained by sucrose-density-gradient centrifugation is close to the value of 6.1S found for the yeast enzyme (Yue *et al.*, 1969) by sedimentation-velocity measurements; this was to be expected, since a commercial preparation of the yeast enzyme sedimented at the same rate in a sucrose gradient (with respect to the cytochrome *c* and catalase markers) as the *P. duponti* enzyme. Sucrose-density-gradient centrifugation in the presence and the absence of $NADP^+$ or glucose 6-phosphate failed to show any change in the sedimentation rate of the *P. duponti* enzyme; this technique had been used by Kirkman & Hendrickson (1962) and Tsutsui & Marks (1962) to demonstrate an $NADP^+$-induced increase in the sedimentation rate of erythrocyte glucose 6-phosphate dehydrogenase.

The stability of the *P. duponti* enzyme was markedly enhanced by the presence of glucose 6-phosphate; no other stabilizing agent could be found. Glucose 6-phosphate also protected the enzyme from inactivation by heat, urea and *p*-chloromercuribenzoate, but $NADP^+$ gave protection only against the latter two agents. These results should be compared with those from studies of glucose 6-phosphate dehydrogenases from yeast (Yue *et al.*, 1969), from erythrocytes (Kirkman & Hendrickson, 1962; Yoshida, 1966; Cohen & Rosemeyer, 1969*a*,*b*), and from cow adrenal

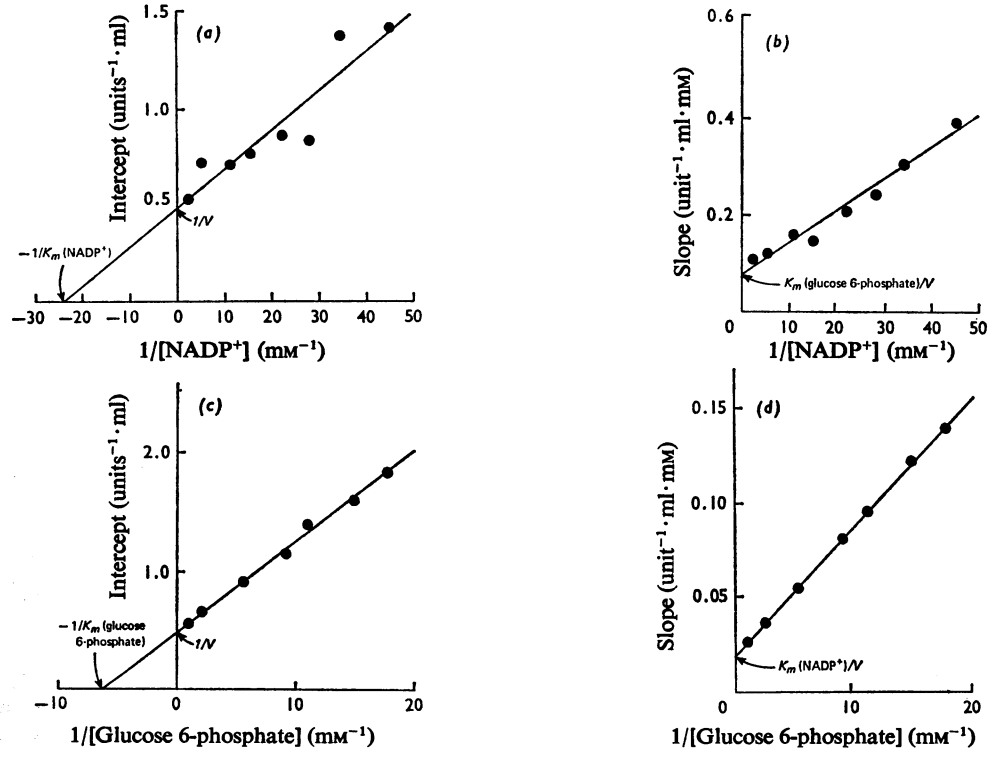

Fig. 7. *Secondary plots of the initial-velocity results for P. duponti glucose 6-phosphate dehydrogenase*

The method for deriving the K_m values is indicated in the figure. The vertical intercept and slope of the lines in Fig. 1(*b*) are plotted against the reciprocal of the $NADP^+$ concentration in Figs. 7(*a*) and 7(*b*) respectively. The vertical intercept and slope of the lines in Fig. 1(*a*) are plotted against the reciprocal of the glucose 6-phosphate concentration in Figs. 7(*c*) and 7(*d*) respectively.

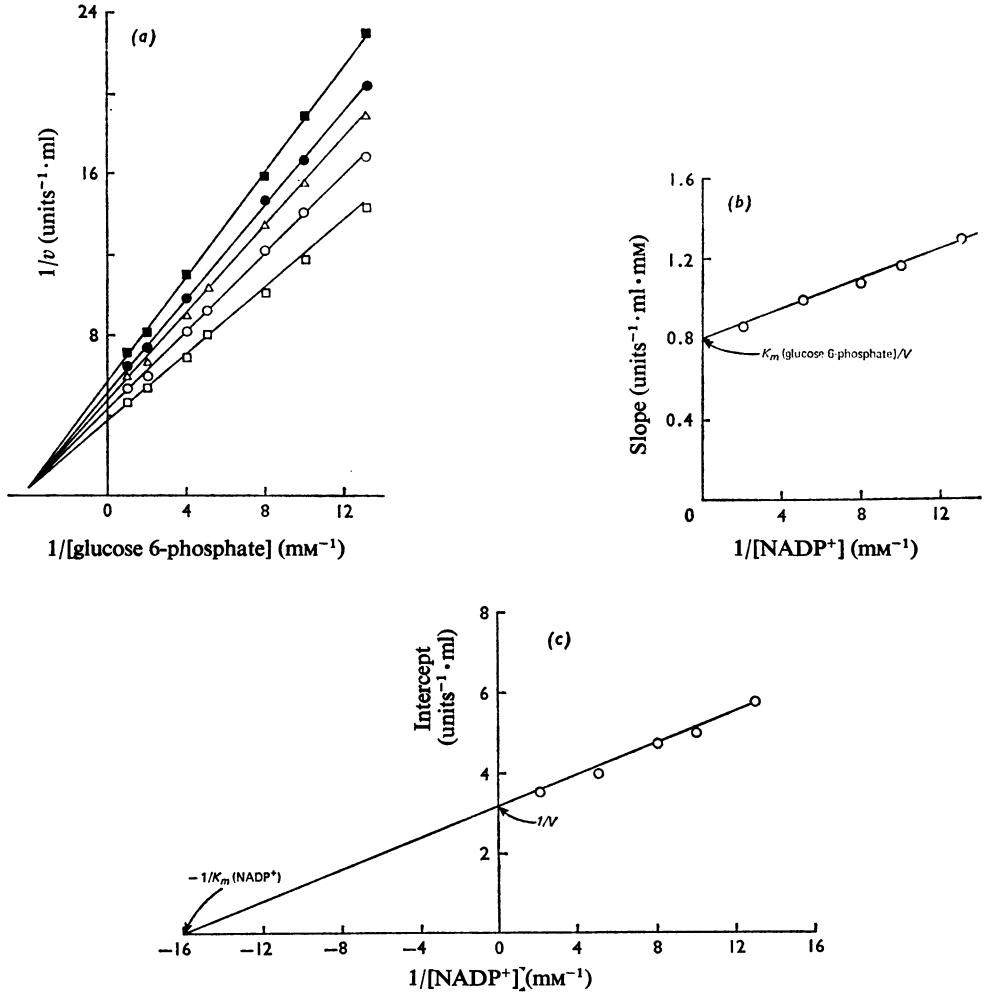

Fig. 8. *Initial-velocity results for P. notatum glucose 6-phosphate dehydrogenase*

The method of deriving the K_m values is indicated in the figure. (*a*) Variation of the initial rate (v) of NADPH production with glucose 6-phosphate concentration at different fixed concentrations of $NADP^+$: □, 0.5mM; ○, 0.2mM; △, 0.125mM; ●, 0.1mM; ■, 77μM. (*b*) Slopes of the lines in (*a*) plotted against the reciprocal of the $NADP^+$ concentration. (*c*) Vertical intercepts of the lines in (*a*) plotted against the reciprocal of the $NADP^+$ concentration.

cortex (Criss & McKerns, 1968) where $NADP^+$ was normally used as a stabilizing agent during purification and crystallization of the enzyme. $NADP^+$ also protected the yeast (Yue *et al.*, 1969) and erythrocyte (Chung & Langdon, 1963) enzymes from *p*-chloromercuribenzoate inactivation. It appears, however, that the stability imparted to these enzymes by $NADP^+$ is due to the role of $NADP^+$ in promoting the association of two dimeric enzyme molecules to form a tetramer, which is more stable than the dimer. Thus the finding that $NADP^+$ does not stabilize the *P. duponti* enzyme supports the result that the enzyme in the presence of $NADP^+$ did not have an increased sedimentation rate on sucrose-density-gradient centrifugation. *P. duponti* glucose 6-phosphate dehydrogenase therefore may not contain the 'structural' $NADP^+$-binding sites which Bonsignore *et al.* (1970) propose in their model for the erythrocyte enzyme. Whether or not this is an adaptation to thermophily has not been investigated, but the

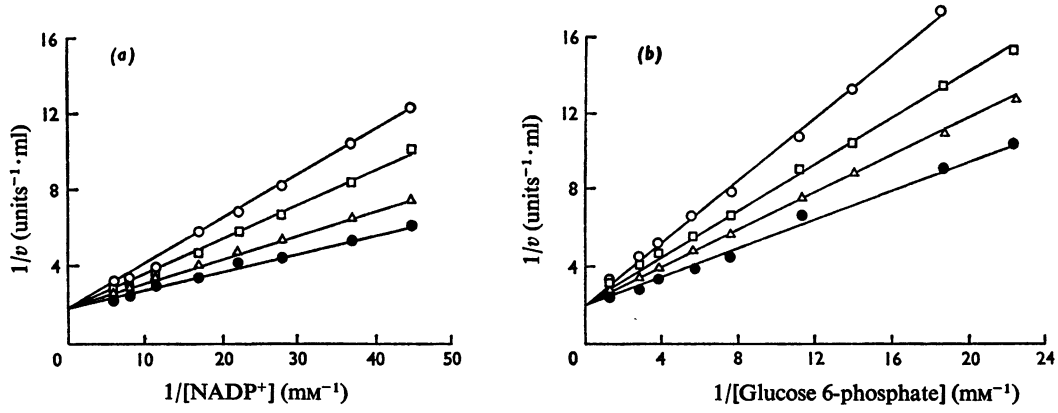

Fig. 9. *Inhibition of P. duponti glucose 6-phosphate dehydrogenase by NADPH*

(*a*) Variation of the initial rate (v) of NADPH production with $NADP^+$ concentration at fixed glucose 6-phosphate concentration (0.36mM-glucose 6-phosphate) and different fixed concentrations of NADPH: ●, no NADPH; △, 8.9μM; □, 20μM; ○, 36μM. (*b*) Variation of the initial rate (v) of NADPH production with glucose 6-phosphate concentration at fixed $NADP^+$ concentration (89μM-$NADP^+$) and different fixed concentrations of NADPH: as in (*a*).

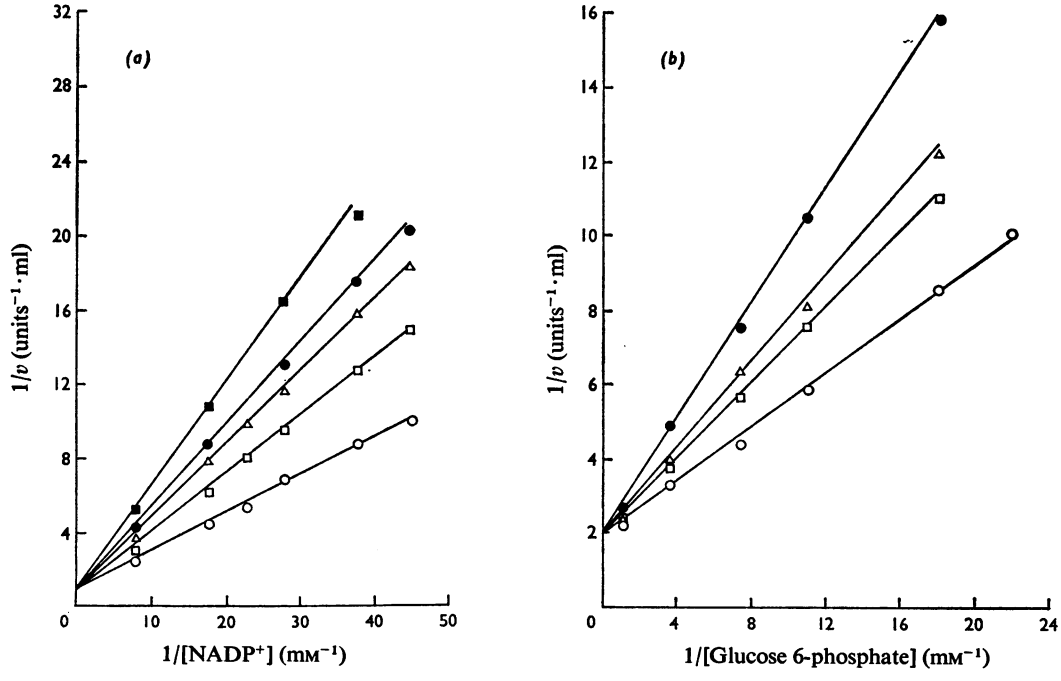

Fig. 10. *Inhibition of P. notatum glucose 6-phosphate dehydrogenase by NADPH*

(*a*) Variation of the initial rate (v) of NADPH production with $NADP^+$ concentration at fixed glucose 6-phosphate concentration (0.36mM-glucose 6-phosphate) and different fixed concentrations of NADPH: ○, no NADPH; □, 8.9μM; △, 20μM; ●, 26μM; ■, 36μM. (*b*) Variation of the initial rate (v) of NADPH production with glucose 6-phosphate concentration at fixed $NADP^+$ concentration (89μM-$NADP^+$) and different fixed concentrations of NADPH: ○, no NADPH; □, 8.9μM; △, 20μM; ●, 36μM.

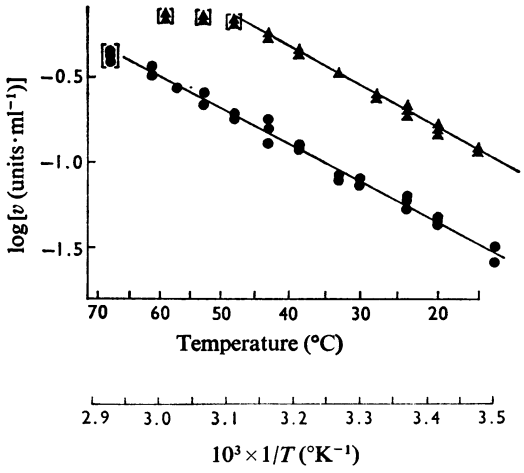

Fig. 11. *Arrhenius plots for P. duponti and P. notatum glucose 6-phosphate dehydrogenases*

The activation energy, E_a, was calculated from the slope of the plot of log (initial velocity) against 1/temperature (°K) which is equal to $E_a/2.303R$, where R is the gas constant. E_a for *P. duponti* enzyme (●) was 40.2kJ·mol^{-1} (9.6kcal·mol^{-1}) and for *P. notatum* enzyme (▲) was 41.4kJ·mol^{-1} (9.9kcal·mol^{-1}).

observation by Cohen & Rosemeyer (1969*a*,*b*) that the association of two dimers to form a tetramer involves ionic bonding supports the suggestion. It is known that ionic interactions become weaker at higher temperatures, so it is probable that in *P. duponti* a dimeric form is more stable.

The erythrocyte enzyme is protected from heat inactivation by low concentrations of NADP$^+$ or high concentrations of glucose 6-phosphate (Marks *et al.*, 1961), and the protection constant (K_s) for glucose 6-phosphate (1.5×10^{-3}M) derived by these workers is similar to that found for the *P. duponti* enzyme (2.25×10^{-3}M). Glucose 6-phosphate dehydrogenase from *Escherichia coli* is also protected from heat inactivation by glucose 6-phosphate, but not by NADP$^+$ (Scott & Cohen, 1953).

When the rates of thermal inactivation of the *P. duponti* and *P. notatum* enzymes were compared, it was found that the thermophile enzyme was much more heat-stable than its mesophile counterpart and this feature was not confined to the two fungi selected for more detailed study (Table 2). In these studies it was important to define the conditions of inactivation carefully, because the rate of inactivation depended to some extent on the specific activity of the enzyme (Fig. 3). The greater thermostability of glucose 6-phosphate dehydrogenase from the thermophile is in accordance with the behaviour of enzymes isolated from thermophilic and mesophilic bacteria (Farrell & Campbell, 1969).

Gaughran (1947) has suggested that a possible reason for the inability of thermophiles to grow at mesophile temperatures might be that thermophile enzymes have a higher energy of activation than mesophile enzymes. This is not supported by our results, however, which show very similar activation energies for the *P. duponti* and *P. notatum* glucose 6-phosphate dehydrogenases, but nor is the proposal eliminated, since the enzyme that is growth-rate-limiting may not be glucose 6-phosphate dehydrogenase.

The thermophile and mesophile enzymes also yielded similar kinetic results. The K_m values for both NADP$^+$ and glucose 6-phosphate were slightly lower for *P. duponti* glucose 6-phosphate dehydrogenase than the values determined for the *P. notatum* enzyme. The double-reciprocal plots of the initial-velocity results had intersecting patterns of lines, clearly ruling out the possibility of a Ping Pong reaction mechanism, and leaving the alternatives of an ordered, Theorell–Chance, or rapid-equilibrium random mechanism (Morrison, 1965). Product inhibition by NADPH was found to be competitive with respect to both NADP$^+$ and glucose 6-phosphate for the *P. duponti* enzyme and for the *P. notatum* enzyme, and the inhibition constants were almost identical. This inhibition pattern is consistent with a rapid-equilibrium random mechanism, which may or may not involve a dead-end enzyme–NADP$^+$–lactone complex (Cleland, 1963), but is incompatible with either an ordered or a Theorell–Chance mechanism. The characteristics of the inhibition by 6-phosphogluconolactone would be desirable for substantiating this conclusion, but could not be obtained under the present conditions, since the half-life of the compound is only about 1.5min in the pH range 7–9 (Horecker & Smyrniotis, 1953).

Soldin & Balinsky (1968) in their studies on erythrocyte glucose 6-phosphate dehydrogenase, found that the NADPH inhibition was competitive with respect to NADP$^+$ and non-competitive with respect to glucose 6-phosphate. This was consistent with either a compulsory-order mechanism in which NADP$^+$ bound first, or a rapid-equilibrium random mechanism with a dead-end enzyme–glucose 6-phosphate–NADPH complex. For a compulsory-order mechanism, the initial-velocity results yield an enzyme–substrate dissociation constant [K_s(A)] for the substrate which binds first (NADP$^+$ in the case of the erythrocyte enzyme), but if the random mechanism holds, the results also allow evaluation of K_s(B) by the equation: $K_s(A)\cdot K_m(B) = K_m(A)\cdot K_s(B)$ (Alberty, 1953). Thus Soldin & Balinsky (1968) decided that the random mechanism could probably be ruled out on the basis of the thermal-inactivation studies of Marks *et al.* (1961), which yielded a K_s

value for $NADP^+$ similar to the K_s(A) value, but a K_s for glucose 6-phosphate 17 times greater than the K_s(B) value. In the case of the *P. duponti* enzyme our results provide no way of distinguishing which substrate binds first (if in fact the mechanism was compulsory-order); however, if we assume that $NADP^+$ does bind first, $K_s(A) = 9.2 \times 10^{-5}$ M, and from the above equation, $K_s(B) = 3.2 \times 10^{-4}$ M. Our results give a dissociation constant for glucose 6-phosphate, K_s(B), which is 7 times smaller than the protection constant, π_g, obtained from the heat-inactivation results [π_g is by definition equivalent to the K_s of Marks *et al.* (1961)]. Therefore, as with the erythrocyte enzyme, a random mechanism can probably be excluded for the *P. duponti* enzyme.

As an alternative to the random mechanism indicated by our kinetic analysis, we would like to suggest that the *Penicillium* glucose 6-phosphate dehydrogenases have a 'normal' compulsory-order catalytic mechanism in which $NADP^+$ will bind preferentially before glucose 6-phosphate, and in addition, a site at which glucose 6-phosphate may bind in the absence of $NADP^+$. This second feature could be a different 'looser' type of binding at the catalytic site, or could involve the binding of glucose 6-phosphate at a separate 'structural' site, the characteristics of which were reflected in the heat-inactivation protection constant.

But the question remains as to why the kinetic and heat-inactivation parameters of the erythrocyte and *P. duponti* enzymes were similar and yet the NADPH-inhibition patterns were different. This could be explained if in the erythrocyte enzyme the glucose 6-phosphate 'protection' binding did not influence NADPH binding to such an extent as in *P. duponti* glucose 6-phosphate dehydrogenase. Thus, in the fungal enzyme, as the glucose 6-phosphate concentrations were raised to values where 'protection' binding became significant, the K_i for NADPH was increased and its inhibiting effect decreased until at infinite glucose 6-phosphate concentration the inhibition was totally overcome. Support for this suggestion comes from the K_i values derived from the kinetic results, when it is noted that higher K_i values were obtained from the plots when glucose 6-phosphate was the variable substrate than when $NADP^+$ was the variable substrate.

Palmitoyl-CoA inhibited *P. duponti* glucose 6-phosphate dehydrogenase in a non-competitive manner and yielded a K_i of 5×10^{-6} M-palmitoyl-CoA. However, the significance of inhibition by compounds containing a lipid moiety is in considerable doubt, as the effect could be attributed to a non-specific detergent-like action (Majeros & Vagelos, 1967). For glucose 6-phosphate dehydrogenase, the inhibition may be related to feedback by the end-product, because of the enzyme's role in providing some of the NADPH required for fatty acid synthesis, although this does not rule out the likelihood of a non-specific interaction.

The *P. duponti* enzyme appeared to have a high degree of both substrate and coenzyme specificity; in fact no alternatives were found. This factor, and also the lack of a substrate analogue that would even compete with the normal substrate, severely limits the scope of further analysis of this enzyme in this state of purity.

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